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Measurement of Fecal Progesterone in Female Dugong (*Dugong dugon*)

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ABSTRACT
In this study, we investigate the possibility of measuring fecal progesterone (P_4) of dugongs in captivity. Fecal samples were collected from a captive adult female dugong kept at Toba aquarium in November 2012 and February 2013, and fecal P_4 of these samples was measured using enzyme immunoassay (EIA). To establish a measuring system, we compared the results of measurements applying different dilution rates of primary antibody and enzyme linked antigen, and decided the best experimental condition for accurate measurement. We then measured P_4 concentrations of the dugong's fecal samples. Significant difference in the P_4 concentrations was not found among the samples collected in November and February. Thus it is suggested the dugong was not in estrous period when the samples were collected. We succeeded in extracting and measuring fecal P_4 of the dugong, and for further study, we plan to apply this method to monitoring of estrous cycle of dugongs.

Keywords: dugong, fecal progesterone, EIA

INTRODUCTION

Dugongs (*Dugong dugon*) are herbivorous marine mammals living in warm and shallow sea. The dugong population is now decreasing in many habitats and they are in need of protection. Reproduction in captivity is one of the most effective methods of preventing the extinction of animals. However, in case of dugongs, captive reproduction has not been successful to date. More information on the estrous cycle is needed to aid reproduction success in captive conditions,

Measurement of a sex hormone is an effective method in understanding the estrous stage of animals. To measure the sex hormone, direct sampling from the target is indispensable. In many case, serums or urine is collected as samples (Wakai *et al*., 2002; Ortiz *et al*., 2000). However, these sampling requires specific trainings for the animals and thus can potentially be stressful for them. Thus, it is important to develop another method without stress. Recently, there are some studies introducing the analysis of fecal hormones to get information on reproduction, such as the state of pregnancy and estrous cycle (Kinoshita *et al*., 2011; Burgess *et al*., 2012). By using feces as samples to measure hormones, we can obtain samples without touching the target animals, because the researcher needs only to collect feces after defecation. Thus this method has little effect on the target animals and can be a good method for monitoring estrous cycle of animals. In a previous study, it was reported that analysis of fecal progesterone (P_4) is effective in diagnosing pregnancy in free-ranging dugongs (Burges *et al.*, 2012). However, there is no study in which a number of fecal samples from the same individual were collected in order to measure fecal hormones.

In this study, first, we established a measuring system of fecal P_4 of dugongs. Then, we obtained fecal samples from a dugong in captivity, and examined the change of fecal P_4 of the particular individual by comparing the results among samples collected in different months.

MATERIALS AND METHODS

Sample collection

This study was conducted in accordance with the Regulation on Animal Experiment at Kyoto University. The target animal of this study was a female dugong, "Serena", living in captivity at Toba aquarium. The animal was 27 years old and had lived in Toba aquarium since 1987. There is no data about what age at dugongs enter menopause. However, dugongs are long-lived animals living up to 73 years, and previously a 42-year old female dugong in the wild was reported to be pregnant with a large foetus (Marsh *et al*., 1984; 2002). Also, Wakai *et al*. (2002) reported that there was cyclic change in Serena's urinary P_4 and the elevation of P_4 was detected periodically. Thus it is suggested that Serena was still capable of breeding, and maintains regular estrous cycle.

Fecal samples were collected from Serena, from November 12 to 14, 2012, and February 7 and 8,

2013. The feces were routinely collected and measured at the end of the day, and we used a part of it as samples. On February 8, we were able to collect samples twice in that day, in the morning (referred to a.m.) and in the afternoon (referred to p.m.). The samples were stored at -35℃ until analysis.

P_4 extraction from fecal samples

The feces were oven-vacuumed at 55℃ for 24 h and subsequently pulverized. Then 0.06g of each sample was vortexed for 30 min in 3 ml of 80% methanol, and centrifuged at 2500 rpm for 10 min.

Enzyme immunoassay

The P_4 concentrations in the supernatant of extracts were analyzed by double-antibody enzyme immunoassay (EIA), according to the methods described by Kinoshita *et al*. (2009). In EIA, we make use of antigen-antibody reactions and color development reactions to measure the concentrations of the target substance. In brief, the target substance, P_4 in this study, acts as an antigen and competes with enzyme linked antigens to bind to its specific antibody, which allows us to detect target substance in samples. The measurement reagents used in this study and their functions are as follows.

Primary antibody:

- P_4 antiserum (FKA302E; Cosmo Bio Co., Ltd., Tokyo, Japan)
- Connects with the second antibody on the plate.

Second antibody:

- Goat anti-rabbit IgG (H+L) (A10533, Life technologies Co., Tokyo, Japan).
- Bind various primary antibodies, including target substance and P_4 antiserum.

Enzyme linked antigen:

- horseradish peroxidase conjugated P_4 (FKA301; Cosmo Bio Co., Ltd.)
- Connects with the same binding site of the primary antibody as the target substance.
- Enzyme linked to the antigen causes color development reactions.

P_4 standard:

- P-8783; Sigma-Aldrich Co., Steinheim, Germany
- Used to make P_4 solutions of known concentrations for obtaining a standard curve.

Chromogenic substrate

- Mixture of equal parts of substrate buffer solution A (0.01-M urea hydrogen peroxide, 0,1-M Na_2HPO_4, 0.05-M citric acid) and solution B (0.002-M 3,3',5,5',-tetra methyl benzidinne, 4% dimethyl sulfoxide, 0.05-M citric acid)
- Combines with enzyme linked antigen and causes color developments.

Because the target substance and the enzyme linked antigen combine with the same binding site of the primary antibody, by adding the target substance and enzyme linked antigen at the same time, they compete to combine with the primary antibody on the plate. The enzyme linked antigens that could combine with the primary antibody cause color developments, thus we can know the concentrations of the target substance by examining the state of color development by measuring absorbance. The concentrations of the target substance in samples were calculated from the absorbance and regression equation obtained from the standard curve. The standard curve was drawn from the absorbance of stepwise diluted solutions of target substance, whose concentrations were known (referred to "standards").

Detailed procedure is described below.

Antigen-antibody reaction

(1) 96-well plate was bound with secondary antibody.

(2) Duplicate 20 µl aliquots of each fecal extract were added to the plate. To obtain the standard curve, P_4 solutions of known concentrations of 0.195-100ng/ml made by diluting P_4 standard with EIA buffer (0.15-M NaCl, 0.04-M Na_2HPO_4, 0.1% bovine serum albumin, pH 7.2) were also added into the wells in duplicate.

(3) 100 µl enzyme linked antigen (2:4000) was added to each well.

(4) 100 µl primary antibody (5.7:16000) was added to each well.

(5) The plates were incubated in the dark for more than 12h at 4℃ and after that, free-bound separation (target substance, primary antibody and enzyme linked antigen) was removed by emptying and washing the plates four times with 0.05% Tween-80 solution.

Color development

(6) 150 µl chromogenic substrate was added to each well, followed by incubation for 15 min at 37℃ in shaking incubator in the dark. Then the color of the solution turned blue, the depth of which was determined by the concentration of P_4 in each solution.

(7) The color development was stopped by the addition of 50 µl of 4-N H_2SO_4.

(8) The absorbance at 450 nm measured using a microplate reader (Sunrise, Rainbow RC-R, Tecan

Table 1 Combination of the dilution rate of primary antibody and enzyme linked antigen

	Primary antibody	Enzyme linked antigen
a	5.7 : 8000	1 : 4000
b	5.7 : 16000	2 : 4000
c	5.7 : 16000	1 : 4000

Japan Co., Ltd, Kanagawa, Japan).

The absorbance for all samples and standards were expressed as the means of duplicated determinations. The P_4 concentrations of fecal samples are indicated as ng/g of dry fecal weight (DFW).

This method differs slightly from a single-antibody EIA, used by Burgess *et al*. (2012), in that it uses two kinds of antibodies. Single-antibody EIA uses only one kind of antibody and can be conducted at lower cost. In the single-antibody EIA, the plate needs to be coated with an antibody that is specific to the focus substance in each experiment, which takes at least 12h of incubation. Double-antibody EIA, on the other hand, uses two kinds of antibodies; a primary antibody and a secondary antibody. The second antibody can bind with various primary antibodies, thus double-antibody EIA can quantify various substances and is easy to prepare. Therefore, we used a double-antibody EIA to save time and enhance effectiveness of the experiment.

Precision of the EIA is affected by the dilution rate of the primary antibody and the enzyme linked antigen. Thus, firstly, we determined the best condition by comparing standard curves applying three different combinations of the dilution rate (Table 1). Secondly, we measured P_4 in samples collected from November 12th to 14th, 2012, and February 7th and 8th, 2013, applying the best condition determined by previous experiments.

RESULTS and DISCUSSION

We examined the best combination of measuring reagent, primary antibody and enzyme linked antigen, by comparing the standard curves applying different conditions. Ideal standard curve requires absorbance at around 1.5, which means moderate color development. A wide range of absorbance is desirable, as well. Considering these requirements, we decided to use condition (b); primary antibody and enzyme linked antigen diluted 5.7:16000 and 2:4000, respectively.

The range of P_4 concentrations of samples collected in November 2012 and February 2013 were 636-850 ng/g and 848-1033 ng/g, respectively. Burges *et al*. (2012) reported that fecal P_4 metabolite (fp) concentrations of female dugongs in wild ranged between 29-8658 ng/g and fp concentrations of confirmed pregnant females (ranging 2017-7760 ng/g) were significantly higher than that of non-pregnant females (ranging 30-221 ng/g). Though we could not perform statistical comparison due to limited number samples, considering this wide range of the concentrations of females and difference between pregnant and non-pregnant females, there seemed to be no difference in the P_4 concentrations among the two months of this study. Also, Wakai *et al*.

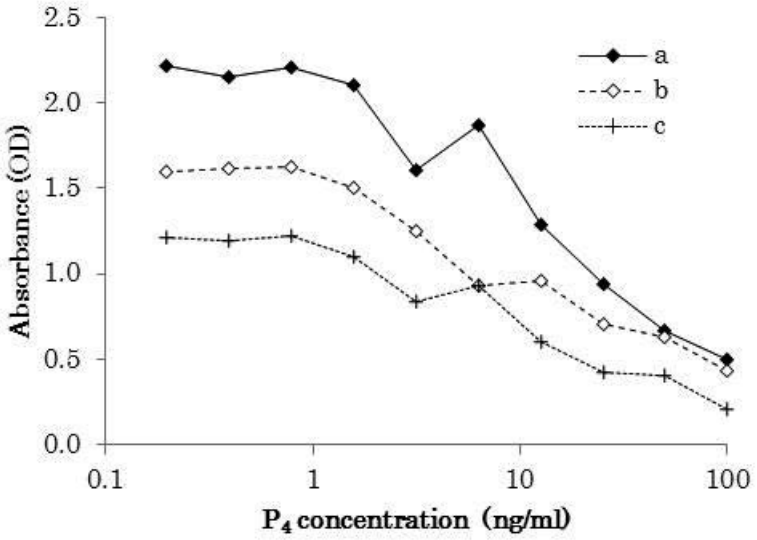

Figure 1 Standard curves of P_4.
The standard curves obtained in three different combinations of primary antibody and enzyme linked antigen are represented by different marks. The horizontal axis indicates P_4 concentrations of standards. The vertical axis shows absorbance (OD: optical density).

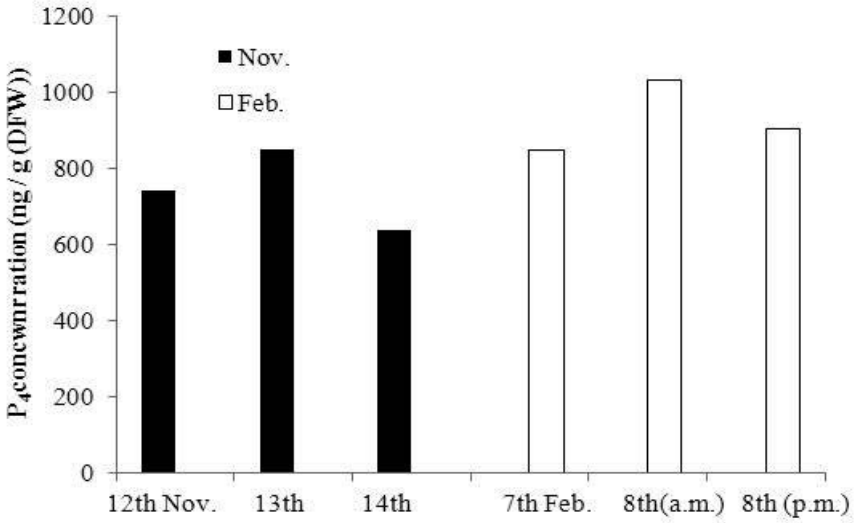

Figure 2 P_4 concentrations of fecal samples. The vertical axis shows the values of P_4 indicated as ng/g of dry fecal weight (DFW) and the horizontal axis shows date of sample collection. On February 8, the samples were obtained twice (a.m. and p.m.) in that day.

(2002) estimated that the length of the dugong's estrous cycle was about 53 days, and the urinary P_4 elevation was detected only for about one or two weeks in the previous study. Thus, considering the results of previous studies, it seems that Serena was not in estrous period when the samples were collected.

In this study, we investigated the possibility of measuring fecal P_4 of dugongs in captivity, applying the EIA method, and succeeded in extracting and measuring fecal P_4. In further studies, we will apply this method to monitoring the estrous cycle of the captive dugong by continuously collecting fecal samples and measuring P_4. By detecting an elevation in P_4, we will be able to know when the target animal is in estrous period.

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